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Increase in heme oxygenase-1 levels ameliorates renovascular hypertension

Fady T. Botros, Michal L. Schwartzman, Charles T. Stier, Jr., Alvin I. Goodman, and Nader G. Abraham

Departments of Pharmacology and Medicine, New York Medical College, Valhalla, New York

Increase in heme oxygenase-1 levels ameliorates renovascular hypertension.

Background. The heme oxygenase system (HO-1 and HO-2) catalyzes the conversion of heme to free iron, carbon monoxide (CO), a vasodepressor, and biliverdin, which is further converted to bilirubin, an antioxidant. HO-1 induction has been shown to suppress arachidonic acid metabolism by cytochrome P450 (CYP450) monooxygenases and cyclooxygenases (COX), and to decrease blood pressure in spontaneously hypertensive rats (SHR). The Goldblatt 2K1C model is a model of renovascular hypertension in which there is increased expression of COX-2 in the macula densa and increased renin release from the juxtaglomerular apparatus of the clipped kidney. We examined whether HO-1 overexpression, as a prophylactic approach, would attenuate renovascular hypertension and evaluated potential mechanisms that may account for its effect.

Methods. 2K1C rats were treated with cobalt protoporphyrin (CoPP) or tin mesoporphyrin (SnMP) one day before surgery and weekly for three weeks thereafter. We measured systolic blood pressure, HO activity, HO-1, HO-2, COX-1 and COX-2 protein expression, heme content, and nitrotyrosine levels as indices of oxidative stress. Urinary prostaglandin excretion (PGE_2), plasma renin activity (PRA), and plasma aldosterone levels were also measured.

Results. CoPP administration induced renal HO-1 expression by 20-fold and HO activity by 6-fold. This was associated with a reduction in heme content, nitrotyrosine levels, COX-2 expression and urinary PGE_2 excretion, and attenuation of the development of hypertension in the 2K1C rats. There was no decrease in plasma renin activity; however, plasma aldosterone levels were significantly lower. In the 2K1C SnMP-treated rats, blood pressure was significantly higher than that of untreated 2K1C rats throughout the study, and the difference in the size of the smaller left clipped kidney compared to the nonclipped right kidney was significantly increased.

Conclusion. These findings define an action of prolonged HO-1 induction to interrupt and counteract the influence of the renin-angiotensin-aldosterone system (RAAS) to increase in blood pressure in the 2K1C model of renovascular hypertension. Multiple mechanisms include a decrease in oxidative stress as indicated by the decrease in cellular heme and nitrotyrosine levels, an anti-inflammatory action as evidenced by a decrease in COX-2 and PGE_2, interference with the action of angiontensin II (Ang II) as evidenced by an increase in PRA in the face of a decrease in PGE_2 and aldosterone, as well as the inhibition of aldosterone synthesis.

Key words: heme oxygenase, hypertension, 2K1C, cyclooxygenase, renin, aldosterone.

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Heme functions as the prosthetic moiety for a number of heme-containing proteins with activities that are critical to vascular and renal function. These include soluble guanylate cyclase, nitric oxide synthase (NOS), and enzymes of the eicosanoid biosynthetic pathways, including cytochrome P450 (CYP450) monooxygenases, thromboxane and prostacyclin synthases, and the heme-dependent cyclooxygenases (COX) [1–5]. Heme oxygenase (HO), as the key enzyme in heme degradation, not only controls cellular levels of heme available for the synthesis of heme proteins, but is also responsible for generating bilirubin, an antioxidant, and the vasodepressor gas, carbon monoxide (CO), from heme. The CO can bind to the heme moiety of heme proteins, causing either enzyme activation or inhibition [3].

To date, two HO isoforms (HO-1 and HO-2), the products of two distinct genes, have been shown to be important in the catabolism of heme in mammals. HO-2, which is constitutively expressed, is localized primarily in the brain, testis, and the vascular endothelium [3, 6, 7]. HO-1 is an inducible isoform with widespread tissue distribution, including liver, kidney, and lung [3]. In earlier studies, we and others demonstrated that overexpression of the HO-1 gene in human, rabbit, and rat endothelial cells not only renders the cells resistant to agents that elicit oxidative stress [3, 8–10], but also enhances cell growth [9, 11, 12] and angiogenesis [13]. A decrease in HO-1 gene expression promotes elevation of cellular heme, a pro-oxidant associated with increased free radicals and cellular lipid peroxidation. In contrast, robust HO-1 overexpression has been shown to increase the antioxidant

properties of the cells, which results in attenuation of oxidative injury and formation of adhesion molecules [14].

Numerous studies have implicated the heme-HO system in the regulation of blood pressure. These studies attribute the blood pressure lowering effect of HO-1 induction to various mechanisms, including decreased production of vasoconstrictor eicosanoids and increased production of CO [2, 15, 16]. CO has been shown to function as a vasodilator [17–21], a stimulator of soluble guanylate cyclase [22], an endogenous modulator of the cGMP signaling system [4], an activator of calcium-activated potassium channels (K_{Ca}) in vascular smooth muscle [23], and an inhibitor of endothelin-1 mediated vasoconstriction [18, 24, 25]. Recent reports have implicated HO in the regulation of renal salt excretion. CO generated by HO-dependent heme catabolism has been shown to stimulate the apical 70-pS K-channel [26]. Inhibition of HO has been shown to decrease sodium and fluid reabsorption in the thick ascending limb of the loop of Henle in the rat [27]. Furthermore, exogenous heme administration has been shown to induce HO-dependent natriuresis and diuresis, which were blocked by pretreatment with SnMP [28]. These reports further underscore the potential importance of HO in the regulation of kidney function.

The Goldblatt 2 kidney 1 clip (2K1C) model is a model for renovascular hypertension [29] characterized by the elevation of oxidants due to activation of the renin-angiotensin-aldosterone system (RAAS). The clipped kidney is responsible for renin release and ultimately for the increase in blood pressure, whereas the nonclipped kidney exhibits subnormal renin content [30]; however, elevated angiotensin II (Ang II) levels cause the nonclipped kidney to exhibit increased renal vascular resistance, leading to impairment of autoregulation [31] and tubuloglomerular feedback. Several studies have shown a relationship between the increase in renin release and an increase in COX-2 expression in the cortical thick ascending loop of Henle and in the region of the macula densa [32–34]. Aldosterone can stimulate COX-2 in the kidney and heart, and has been reported to induce vascular inflammatory phenotypes [35–37].

The objective of this study was to examine the consequences of HO-1 induction and HO inhibition on the renal heme-HO system, oxidant levels, and blood pressure in the 2K1C renovascular hypertension model. We measured systolic blood pressure, HO activity, HO-1, HO-2, COX-1 and COX-2 protein expression, heme content, and nitrotyrosine levels as indices of oxidative stress. Urinary prostaglandin (PGE_2) excretion, plasma renin activity (PRA), and plasma aldosterone levels were also measured. Our results demonstrated that basal levels of the constitutively expressed HO-2 were not increased, but heme content and nitrotyrosine levels were increased in the 2K1C model compared to sham animals. Administration of cobalt protoporphyrin (CoPP), a potent inducer of HO-1 protein and activity, attenuated the development of hypertension, possibly by decreasing cellular heme and oxidant levels and/or by increasing the production of CO and bilirubin. Inhibition of HO activity by tin mesoporphyrin (SnMP) magnified oxidative stress, thus, substantiating a significant role for HO-1 as part of the cellular defense system against oxidant damage and maintaining renal hemodynamic function in the 2K1C model. These findings also suggest that there is a significant link between the heme-HO system and the levels of heme-dependent oxidants in the development of hypertension in 2K1C rats.

METHODS

Animal treatment

Male Sprague-Dawley (SD) rats (200–250 g body weight; Charles River Lab, Wilmington, MA, USA) were housed 4 to 5 days before the beginning of the study. Tail blood pressure was measured without anesthesia, using the tail cuff method, at least twice before the surgery. Animals were anesthetized with pentobarbital, and a U-shaped silver clip with an internal gap of 0.25 mm was placed around the left renal artery. Four groups of rats were studied: sham operated animals, 2K1C control rats, 2K1C rats pretreated with CoPP (5 mg/100 g body wt) 24 hours before surgery, and 2K1C rats pretreated with SnMP (5 mg/100 g body wt) 24 hours before surgery. Each group contained 18 animals; a total of 72 rats were used.

Weekly injections of CoPP (5 mg/100 g body wt) were administered subcutaneously to the CoPP group. SnMP (5 mg/100 g body wt) was administered intraperitoneally three times a week to the SnMP group for three weeks. Sham and control 2K1C rats were injected with saline. There was no mortality in the rats with the use of various drugs including CoPP and SnMP. Blood pressure was measured twice a week for three weeks. Blood samples were collected in K_3EDTA tubes, centrifuged at 2500*g* for 10 minutes at 4°C to separate the plasma, which was stored at −20°C. On the last day before sacrifice, rats were housed in metabolic cages for urine collection. Rats were sacrificed on day 21 after the surgery.

Tissue preparation

Kidneys were immediately excised, weighed and frozen at −80°C until use. Tissues were homogenized (4 mL/g wet weight) in buffer, pH 7.4, containing 0.25 mol/L sucrose. The homogenate was centrifuged at 10,000*g* for 10 minutes at 4°C. The cell-free homogenate (10,000*g* supernatant) was used for Western blot analyses or activity assays. Protein concentration was determined according to the method of Bradford and Marshal (Bio-Rad, Hercules, CA, USA).

Western blot analysis

Western blot analysis of HO-1, HO-2, COX-1, and COX-2 proteins was performed as previously described [2]. Cell-free homogenates (10,000*g* supernatant) of kidney preparations (20 μg protein) were separated by sodium dodecyl sulfate (SDS)/polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane using a semidry transfer apparatus (Bio-Rad). The membranes were incubated with 5% milk in 10 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 0.05% Tween 20 (TBST) buffer at 4°C overnight. After washing with TBST, the membranes were incubated for 1 hour with rabbit anti-rat HO-1 or HO-2 polyclonal antibodies (1:1000; Stressgen Biotechnologies Corp., Victoria, BC, Canada) or rabbit antimouse COX-1 or COX-2 polyclonal antibodies (1:250; Cayman Chemical, Ann Arbor, MI, USA). The filters were then washed and subsequently probed with horseradish peroxidase–conjugated donkey antirabbit IgG for HO-1, HO-2, COX-1, or COX-2. Chemiluminescence detection was performed with the Amersham ECL detection kit (Amersham, Piscataway, NJ, USA) according to the manufacturer's instructions.

Measurement of HO activity and heme content

Tissue segments were homogenized (4 mL/g wet weight) in homogenization buffer (pH 7.4), containing 0.25 mol/L sucrose. The homogenates were centrifuged at 10,000*g* for 10 minutes at 4°C. The supernatant was then used for measuring HO activity. HO activity was assayed [2] in which bilirubin, the end product of heme degradation, was extracted with chloroform and its concentration was determined spectrophotometrically (Dual UV/VIS Beam Spectrophotometer Lambda 25; Perkin-Elmer, Norwalk, CT, USA) using the difference in absorbance at wavelength from λ 460 to λ 530 nm with an absorption coefficient of 40 mmol/L^{-1} and cm^{-1}.

Heme content was determined by the pyridine hemochromogen method [2]. The absorbance difference between λ 557 and λ 530 nm was used to calculate the heme content using an extinction coefficient of 20.7 mmol/L^{-1} and cm^{-1}.

Measurement of nitrotyrosine

Nitrotyrosine is a marker for peroxynitrite-mediated oxidative stress and has been regarded as a sensitive marker of oxidative damage. Nitrotyrosine residues were assessed by Western blot analysis with a rabbit anti-nitrotyrosine antibody (Cayman Chemical).

Urinary PGE_2 excretion

Urinary excretion rate for PGE_2 was determined using an ELISA (EIA) kit (Cayman Chemical).

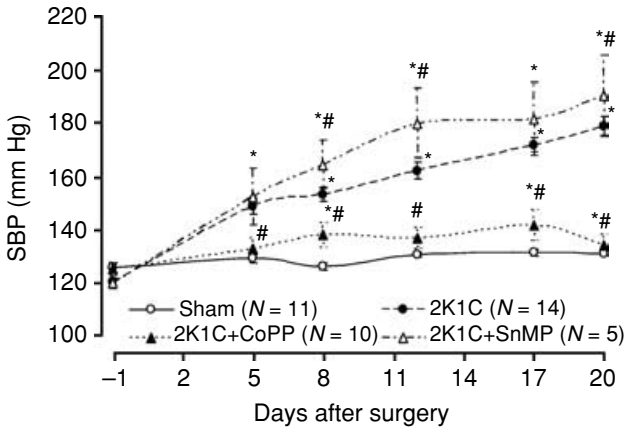

Fig. 1. Effect of treatment with CoPP (5 mg/100 g body wt/week) or SnMP (three doses of 5 mg/100 g body wt/week) for three weeks on systolic blood pressure in 2K1C rats. Results are expressed as mean ± SE for each group. $^*P < 0.05$ vs. sham, $\#P < 0.05$ vs. 2K1C.

Plasma renin activity and aldosterone levels

PRA was determined using commercially available radioimmunoassay (RIA) kits (Perkin Elmer Life Sciences, Boston, Massachusetts, USA; or DiaSorin, Stillwater, MN, USA). Briefly, frozen plasma samples were thawed on ice, diluted with maleate buffer (pH 6.0), and incubated with exogenous renin substrate (plasma from 48-hour nephrectomized rats) in the presence of dimercaprol and 8-hydroxyquinoline for 1 hour at 37°C. RIA was performed to measure Ang I produced in the incubation medium. Plasma aldosterone levels were determined with a commercially available RIA kit (Diagnostic Products Corp., Los Angeles, CA, USA).

Statistical analysis

Results are presented as mean ± standard error (SE) for the number (N) of replicate determinations. Statistical significance between the experimental groups was determined by using one-way analysis of variance (ANOVA) followed by the Fisher least significant difference (LSD) multiple-comparison test; $P < 0.05$ was considered significant. Blood pressure measurements were analyzed by using repeated measures ANOVA followed by the Fisher LSD multiple-comparison test; $P < 0.05$ was considered significant.

RESULTS

Effect of HO expression on development of hypertension

In the sham rats, blood pressure did not change throughout the study. However, in the 2K1C rats, blood pressure was significantly increased by day five and remained significantly elevated. Twenty days post clipping, systolic blood pressure in the untreated 2K1C rats was significantly higher than that of the sham rats (179 ± 4 mm Hg, $N = 14$ vs. 131 ± 1 mm Hg, $P < 0.05$) (Fig. 1). Relative

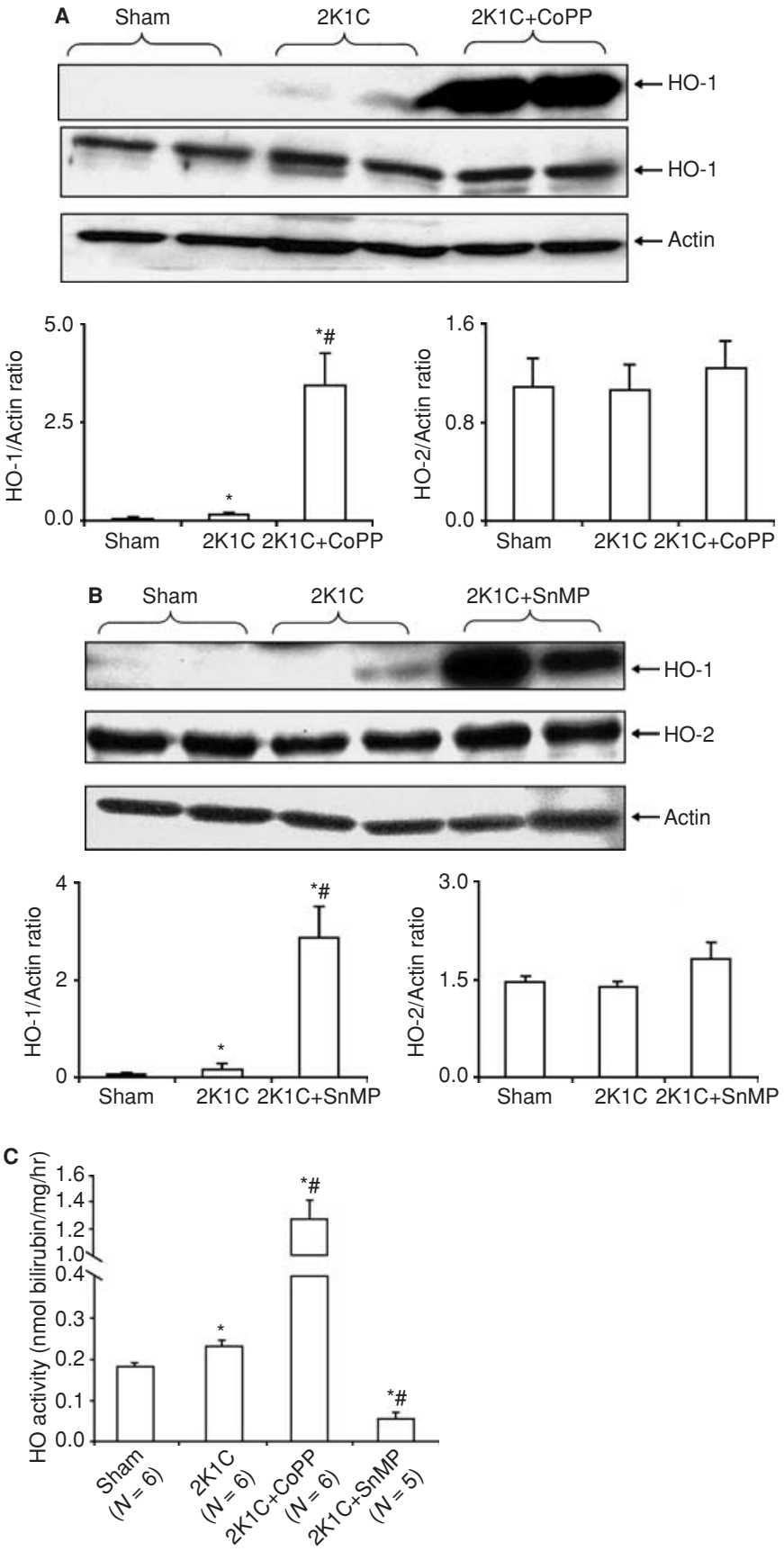

Fig. 2. (*A*) Western blot and densitometry analysis showing the effect of treatment with CoPP (5 mg/100 g body wt/week) for three weeks on HO-1 and HO-2 protein expression in the clipped kidney of 2K1C rats ($N = 6$). (*B*) Western blot and densitometry analysis showing the effect of treatment with SnMP (three doses of 5 mg/100 g body wt/week) for three weeks on HO-1 and HO-2 protein expression in the clipped kidney of 2K1C rats ($N = 3$). (*C*) The effect of treatment with CoPP (5 mg/100 g body wt/week) or SnMP (5 mg/100 g body wt/week) for three weeks on HO activity in the clipped kidney of 2K1C rats. Results are expressed as mean ± SE. $^*P < 0.05$ vs. sham, $\#P < 0.05$ vs. 2K1C.

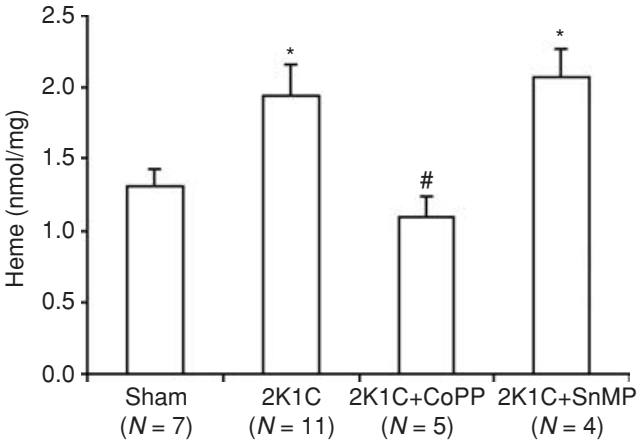

Fig. 3. Effect of treatment with CoPP (5 mg/100 g body wt/week) or SnMP (three doses of 5 mg/100 g body wt/week) for three weeks on heme content in the clipped kidney of 2K1C rats. Results are expressed mean ± SE. *$P < 0.05$ vs. sham, #$P < 0.05$ vs. 2K1C.

to sham rats, treatment with CoPP, a potent inducer of HO-1 protein and activity, markedly attenuated the development of hypertension. Twenty days after clipping, systolic blood pressure in the CoPP-treated 2K1C rats was significantly lower than that of the untreated 2K1C rats (134 ± 4 mm Hg vs. 179 ± 4 mm Hg, $P < 0.05$), and was not different than that of the sham rats (134 ± 4 mm Hg vs. 131 ± 1 mm Hg) (Fig. 1). To examine the effect of HO inhibition on the development of hypertension, we used the potent HO activity inhibitor, SnMP. Twenty days post clipping, systolic blood pressure in SnMP-treated 2K1C rats was significantly higher than that of the untreated 2K1C rats (190.2 ± 15.3 mm Hg vs. 179.0 ± 3.5 mm Hg, $P < 0.05$) (Fig. 1).

Effect of HO inducers and inhibitors on the levels of HO-1 and HO-2

The increase in blood pressure in 2K1C rats was also associated with an increase in clipped kidney HO-1 expression and HO activity. As seen in Figure 2A, HO-1 expression was significantly increased in clipped kidneys from 2K1C rats compared to kidneys from sham rats; however, Western blot analysis revealed no significant difference in HO-2 protein expression (Fig. 2B). Plotting the ratio of HO-1/actin to document the relative increase in HO-1 showed that HO-1 protein was not significantly increased in the 2K1C (sham vs. 2K1C). Renal HO activity was significantly higher in clipped kidneys from 2K1C rats compared to sham rats (0.230 ± 0.016 nmol bilirubin/mg/hr vs. 0.182 ± 0.01 nmol bilirubin/mg/hr, $N = 6$, $P < 0.05$) (Fig. 2C).

Treating 2K1C rats with CoPP increased HO-1 protein expression by 20-fold (Fig. 2A); in contrast, HO-2 protein levels did not change (Fig. 2A). There was also a 6-fold increase in HO activity in clipped kidneys from CoPP-treated 2K1C rats versus untreated 2K1C rats (1.261 ± 0.135 nmol bilirubin/mg/hr vs. 0.330 ± 0.016 nmol bilirubin/mg/hr, $N = 6$, $P < 0.05$) (Fig. 2C). Untreated 2K1C rats have a minimally significant increase in HO-1, whereas 2K1C-CoPP has a highly significant increase in HO-1 protein. Relative to untreated 2K1C rats, a significant increase in HO-1 protein expression was detected in the SnMP-treated 2K1C rats (Fig. 2B); the similarity of the SnMP structure to CoPP resulted in gene activation of HO-1. Although SnMP, like CoPP, is an inducer of HO-1 protein expression, it is also a potent inhibitor of HO activity and is used clinically to control hyperbilirubinemia [38]. SnMP-treated 2K1C rats had significantly lower renal HO activity (0.0268 ± 0.004 nmol bilirubin/mg/hr, $N = 5$, $P < 0.05$) (Fig. 2C). CoPP and SnMP increased HO-1 proteins in the unclipped kidney (data not shown).

Effect of heme-HO system on renal heme

Clipped kidney homogenate from 2K1C rats showed significantly higher levels of heme than that from sham rats (1.93 ± 0.23 nmol/mg protein vs. 1.31 ± 0.12 nmol/mg protein, $P < 0.05$) (Fig. 3). Heme content was significantly lower in CoPP-treated 2K1C rats compared to untreated 2K1C rats (1.10 ± 0.13 nmol/mg protein, $N = 5$ vs. 1.93 ± 0.23 nmol/mg protein, $P < 0.05$) (Fig. 3). Heme content in the clipped kidney tissue homogenate of SnMP-treated 2K1C rats was not significantly different from that of untreated 2K1C rats (2.07 ± 0.20 nmol/mg protein vs. 1.93 ± 0.23 nmol/mg protein) (Fig. 3).

Effect of HO inducers and inhibitors on nitrotyrosine levels

Because peroxynitrite has been shown to be increased in renovascular hypertension [39, 40], we measured nitrotyrosine levels using Western blot analysis. Relative to sham rats, clipped kidneys from 2K1C rats showed significantly higher levels of nitrotyrosine (Fig. 4). The increased HO activity and decreased heme content in CoPP-treated 2K1C rats were associated with significant decrease in the levels of nitrotyrosine immunoreactivity in the clipped kidney (Fig. 4). Treating 2K1C rats with SnMP significantly increased clipped kidney nitrotyrosine levels (Fig. 4). These results demonstrate that clipped kidney tissue from 2K1C rats had a higher level of oxidative stress, which was decreased with CoPP, but increased with SnMP, indicating that induction of HO-1 activity provided protection against oxidative stress.

Effect of HO inducers and inhibitors on COX-1 and COX-2 and urinary PGE_2

Western blot analysis of clipped kidney homogenate revealed significantly higher levels of COX-2 protein expression compared to sham kidneys (Fig. 5A). In contrast,

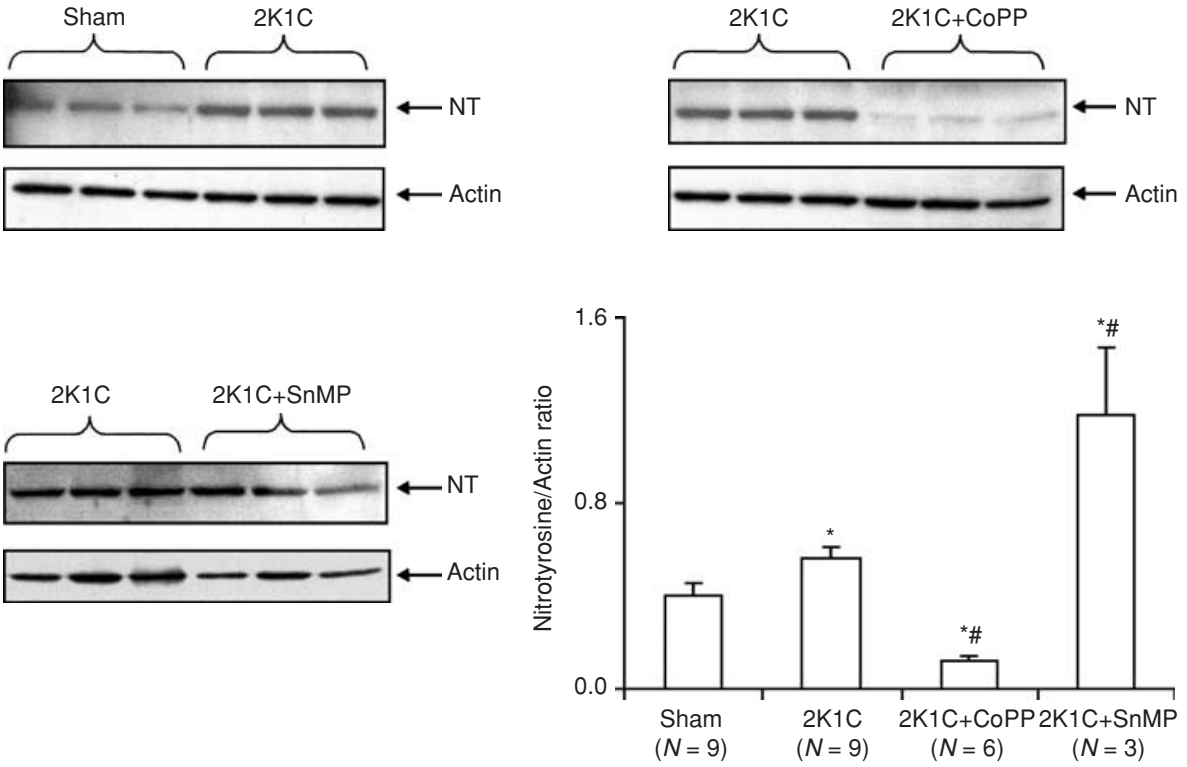

Fig. 4. Western blot and densitometry analysis showing the effect of treatment with CoPP (5 mg/100 g body wt/week) for three weeks or SnMP (three doses of 5 mg/100 g body wt/week) on nitrotyrosine (NT) levels in the clipped kidney. Results are expressed mean ± SE. *$P < 0.05$ vs. sham, #$P < 0.05$ vs. 2K1C.

significant COX-1 levels were detected both in sham kidneys and in clipped kidneys from untreated 2K1C rats (Fig. 5B). Three weeks after clipping, urinary PGE_2 excretion was significantly higher in 2K1C rats versus sham rats (83 ± 18 ng PGE_2/24 hr vs. 47 ± 4 ng PGE_2/24 hr, $P < 0.05$) (Fig. 5C). Compared to untreated 2K1C rats, treatment with CoPP significantly decreased COX-2 levels in the clipped kidney (Fig. 5A); in contrast, COX-1 protein levels did not change (Fig. 5B). In the CoPP-treated 2K1C rats, urinary PGE_2 excretion was significantly lower than that of untreated 2K1C (9 ± 5 ng PGE_2/24 hr vs. 83 ± 18 ng PGE_2/24 hr, $P < 0.05$) (Fig. 5C). Clipped kidney COX-2 expression was not significantly higher in SnMP treated 2K1C than that of the untreated 2K1C rats, and COX-1 protein levels did not change in clipped kidneys from SnMP-treated 2K1C rats compared to untreated 2K1C rats (Fig. 5). In addition, treatment with SnMP did not significantly affect the levels of urinary PGE_2 excretion (Fig. 5C). Since COX-2 was decreased, but not COX-1, this would suggest that the major portion of urinary PGE_2 excretion in 2K1C rats is COX-2–derived.

Effect of heme-HO expression on plasma renin activity

PRA was significantly higher in 2K1C rats compared to sham rats (147 ± 16 ng Ang I/mL/hr vs. 50 ± 11 ng Ang I/mL/hr, $P < 0.05$) (Fig. 6). Although CoPP treatment caused a significant decrease in blood pressure, there was a significant increase in PRA compared to untreated 2K1C (Fig. 6). Treating 2K1C rats with SnMP did not have a significant effect on PRA, although it was higher compared to untreated 2K1C (268 ± 53 ng Ang I/mL/hr, $N = 4$ vs. untreated 2K1C) (Fig. 6).

Effect of HO expression on plasma aldosterone levels

Plasma collected from 2K1C rats showed significantly higher levels of aldosterone compared to sham rats (613 ± 72 pg/mL vs. 307 ± 49 pg/mL, $P < 0.05$) (Fig. 7). Although CoPP increased PRA more than 2-fold higher than that in the untreated 2K1C rats, plasma aldosterone levels were significantly lower than that of the untreated 2K1C rats (411 ± 59 pg/mL vs. 613 ± 72 pg/mL, $P < 0.05$) (Fig. 7). Plasma aldosterone levels were significantly higher in rats treated with SnMP than those of the sham rats (552 ± 12 pg/mL vs. 307 ± 49 pg/mL, $P < 0.05$), but were not significantly different from the untreated 2K1C rats (Fig. 7).

Effect of HO expression on relative size of the kidney, clipped (L) versus non-clipped (R)

As seen in Table 1 and as expected, the relative size of the two kidneys (clipped/non-clipped) was significantly decreased in the 2K1C rats. However, up-regulation of

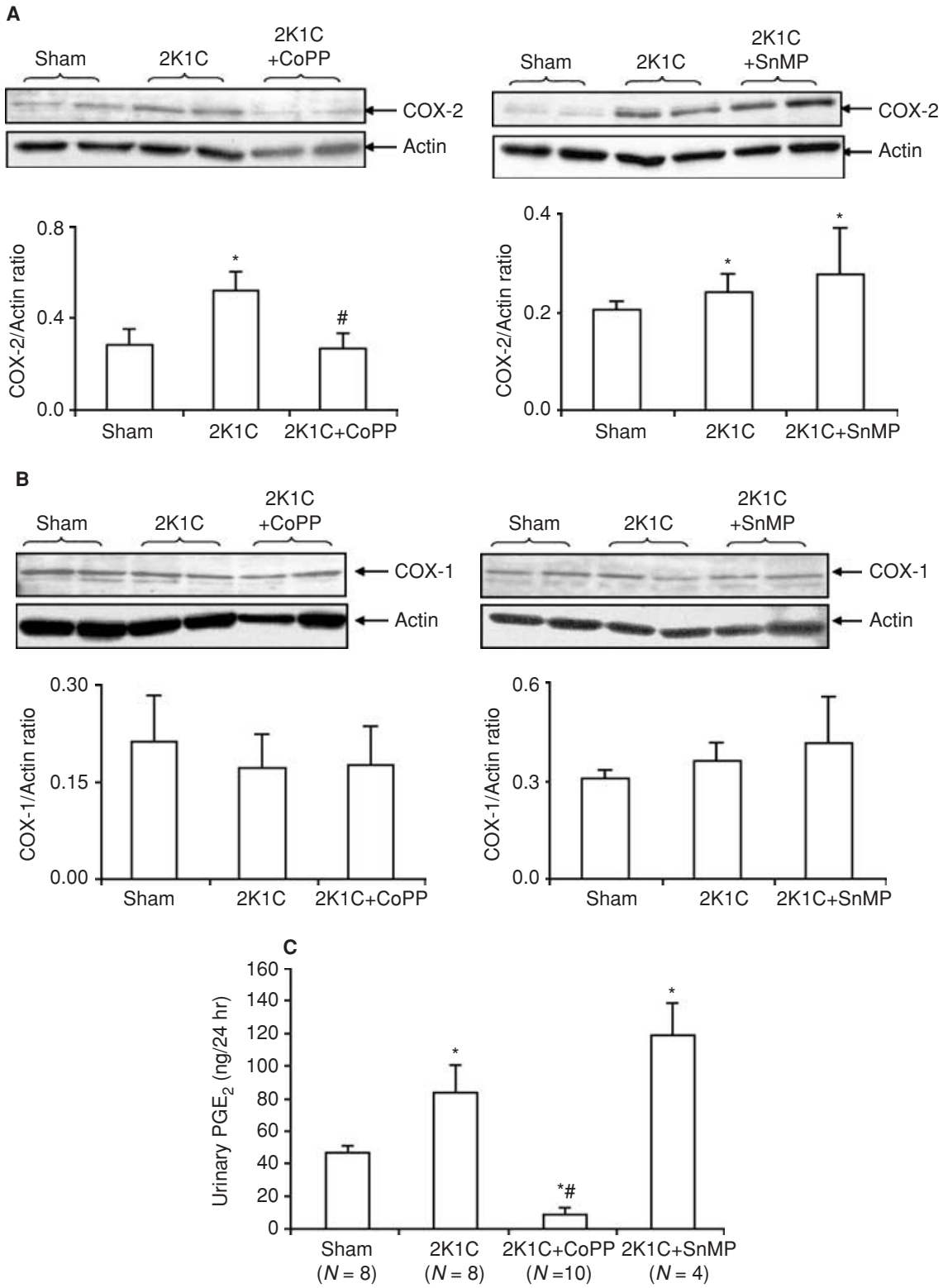

Fig. 5. (*A*) Western blot and densitometry analysis showing the effect of treatment with CoPP (5 mg/100 g body wt/week) ($N = 11$) or SnMP (three doses of 5 mg/100 g body wt/week) ($N = 3$) for three weeks on COX-2 protein expression in the clipped kidney of 2K1C rats. (*B*) Western blot and densitometry analysis showing the effect of treatment with CoPP (5 mg/100 g body wt/week) ($N = 9$) or SnMP (three doses of 5 mg/100 g body wt/week) ($N = 3$) for three weeks on COX-1 protein expression in the clipped kidney of 2K1C rats. (*C*) The effect of treatment with CoPP (5 mg/100 g body wt/week) or SnMP (three doses of 5 mg/100 g body wt/week) for three weeks on urinary PGE_2 excretion in 2K1C rats. Results are expressed mean ± SE. $^*P < 0.05$ vs. sham, $\#P < 0.05$ vs. 2K1C.

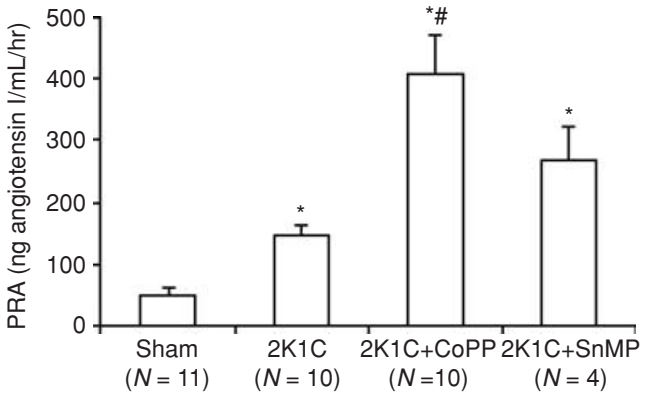

Fig. 6. Effect of treatment with CoPP (5 mg/100 g body wt/week) or SnMP (three doses of 5 mg/100 g body wt/week) on plasma renin activity in 2K1C rats. Results are expressed mean ± SE. $^*P < 0.05$ vs. sham, $\#P < 0.05$ vs. 2K1C.

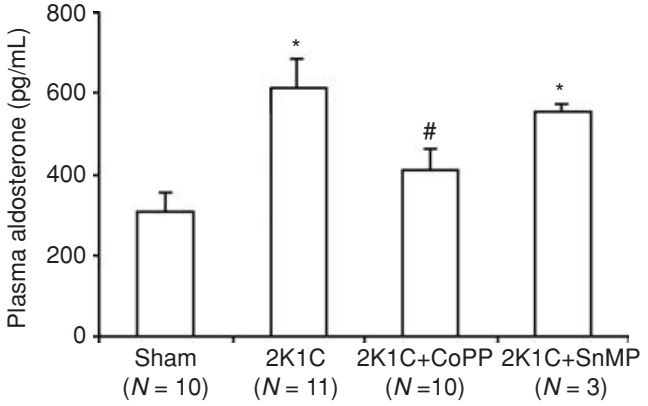

Fig. 7. Effect of treatment with CoPP (5 mg/100 g body wt/week) or SnMP (three doses of 5 mg/100 g body wt/week) on plasma aldosterone levels in 2K1C rats. Results are expressed mean ± SE. $^*P < 0.05$ vs. sham, $\#P < 0.05$ vs. 2K1C.

HO-1 and HO activity by CoPP significantly improved the clipped/non-clipped (L/R) kidney weight ratio, indicating possible increased preservation of kidney tissue. In contrast, inhibition of HO by SnMP magnified the effect of renal artery clipping on kidney size ($P < 0.05$) and decreased the size of the clipped kidney compared to untreated 2K1C. Figure 8 illustrates the effect of inhibition and induction of HO-1 using SnMP and CoPP, respectively, on the size of the clipped kidney, demonstrating the differential effect of HO-1 levels and HO activity on renal cytoprotection. The increase in HO activity by CoPP caused a 65% increase in clipped kidney size compared to the effect of inhibition of HO activity by SnMP ($P < 0.0001$).

DISCUSSION

In this report, we have demonstrated that induction of HO-1 lowers blood pressure in the 2K1C model of renovascular hypertension, whereas inhibition of HO activity increases hypertension. The increase and decrease in blood pressure as a result of the increase and decrease in HO-1, respectively, is related to renal heme content. Heme, a known prooxidant, has been shown to contribute to the generation of reactive oxygen species (ROS) [39, 41, 42], and increased amounts of heme have been shown to cause renal injury [43, 44]. In our study, the increase in cellular heme may constitute a major factor in the increased superoxide-mediated conversion of nitric oxide (NO) to peroxynitrite and the increase in oxidative stress indices observed as an increase in nitrotyrosine immunoreactivity. The increase in heme levels may also be a reflection of the increased levels of heme proteins, including COX-2, which was significantly increased in the clipped kidneys from untreated 2K1C rats. Additionally, COX activity was increased, as indicated by an increase in urinary PGE_2. These results are in agreement with other reports suggesting that the COX pathway is activated in 2K1C hypertensive rats as evidenced by an increase in urinary excretion of both 6-keto-$PGF_{1\alpha}$ and TXB_2 [45]. Other reports have shown that macula densa COX-2 is up-regulated in high-renin states (i.e., as a consequence of low sodium intake [32] or renal artery stenosis [46]) and following inhibition of RAAS [47]. COX-2 plays an important role in the regulation of renin release. Wang et al [34] have shown that COX-2 inhibition decreases renin production and release in renovascular hypertension. However, a report by Hartner et al [33] demonstrated that COX-2 inhibition in established renovascular hypertension does not affect renin synthesis or release, but only partially inhibits the increase in PRA induced by a low salt diet.

Treatment of 2K1C rats with CoPP significantly induced HO-1 expression and total HO activity and was associated with a significant decrease in cellular heme, oxidant levels, COX-2 expression in the clipped kidney, and urinary PGE_2 excretion. We have shown that CoPP significantly increased HO-1 protein expression in the cortex and outer medulla and, to a lesser degree, in the inner medulla of the rat kidney and increased HO activity in both cortex and outer medulla [48]. HO-1 induction by CoPP treatment greatly attenuated the development of hypertension and improved the clipped/non-clipped (L/R) kidney weight ratio (Table 1), suggesting amelioration of renal hypertrophy. This effect was likely linked to the HO-1–mediated decrease in heme content with CoPP. In contrast, treatment with SnMP, which increased heme content as a result of the decrease in HO activity, worsened renal atrophy in 2K1C rats. These results suggest that one of the mechanisms by which HO-1 modulation affects kidney size in 2K1C is via control of the cellular heme pool.

Another mechanism by which up-regulation of HO-1 decreases blood pressure may be related to an increase in the level of CO and bilirubin, a potent antioxidant.

Table 1. Effect of treatment with CoPP (5 mg/100 g body wt/week) or SnMP (three doses of 5 mg/100 g body wt/week) on left (L, clipped)/right (R, non-clipped) kidney weight ratio in 2K1C rats

	Sham	2K1C	2K1C+CoPP	2K1C+SnMP
L. kidney (clipped) *g*	1.277 ± 0.04 ($N = 11$)	1.084 ± 0.06[a] ($N = 13$)	1.259 ± 0.05[b] ($N = 12$)	0.712 ± 0.17[a,b] ($N = 5$)
R. kidney (non-clipped) *g*	1.296 ± 0.05 ($N = 11$)	1.499 ± 0.05[a] ($N = 13$)	1.494 ± 0.05[a] ($N = 12$)	1.441 ± 0.04 ($N = 5$)
L/R kidney weight ratio	0.989 ± 0.01 ($N = 11$)	0.732 ± 0.04[a] ($N = 13$)	0.846 ± 0.02[a,b] ($N = 12$)	0.487 ± 0.12[a,b] ($N = 5$)

Results are expressed as mean ± SE.
[a] $P < 0.05$ vs. sham; [b] $P < 0.05$ vs. 2K1C.

These products may contribute to the renal cytoprotection in non-clipped kidney, where HO-1 is up-regulated due to the increase in circulating Ang II and other inflammatory cytokines. Bilirubin can scavenge ROS [49–51] and inhibit activation of NADPH oxidase [52] and protein kinase C (PKC) [53], which are key signaling steps in oxidant-induced vascular injury. In humans, bilirubin levels have been increasingly shown to be related to cardiovascular disease [54, 55]. Selective inhibition of biliverdin reductase and the consequent reduction in bilirubin has been shown to increase ROS by 3-fold [56]. Therefore, increased HO-derived bilirubin may play a significant role in decreasing oxidants in the 2K1C. In support of this, recent studies have shown increased oxidative stress in the Goldblatt 2K1C model [57], and that treatment with the superoxide dismutase mimetic, tempol, normalized the levels of oxidative stress and lowered blood pressure [58]. Our study provides evidence that heme and bilirubin might play an important regulatory role in oxidative stress. Targeting renal tissues with the human HO-1 gene has also been recently shown to increase antioxidant molecules, such as glutathione [59].

Since elevated plasma renin contributes to the development of hypertension in the 2K1C model, we examined the effect of CoPP on PRA. To our surprise, CoPP did not decrease PRA in the 2K1C rats, but rather increased it. Ploth et al [60] have shown that acute treatment of 2K1C rats with verapamil, an antihypertensive calcium channel blocker, significantly reduced blood pressure and also increased PRA. In view of the fact that induction of HO-1 by CoPP results in the generation of CO, and that CO has been shown to promote vasorelaxation through multiple mechanisms [61], the increased levels of PRA in the CoPP-treated 2K1C rats may be in response to the vasorelaxation and decrease in blood pressure mediated by the effects of CO.

Despite the increased PRA, plasma aldosterone levels in the CoPP-treated 2K1C rats were significantly lower than in untreated 2K1C rats. These results demonstrate that induction of HO-1 by CoPP decreases aldosterone synthesis. HO has been implicated as a major regulator of CYP450 hemoproteins by limiting the amount of heme and/or by producing CO, which strongly binds to the heme moiety of CYP450 causing enzyme inhibition [3]. Therefore, by limiting the availability of heme or by increasing CO production, HO may inhibit the P450 enzymes required for aldosterone synthesis (P450scc and P450 aldosterone synthase) and, consequently, reduction in aldosterone synthesis may favor, at least in part, the lowering of blood pressure observed in the CoPP-treated 2K1C rats. Another possible explanation is that induction of HO-1 and the resulting decreased heme levels may interfere with the action of Ang II in 2K1Cl rats, which would explain the increase in PRA and decrease aldosterone levels. However, these new observations remain to be investigated, including the possibility that HO-1 induction may act as an AT_1 receptor blocker. We have previously shown that targeting the thick ascending limb of the loop of Henle with human HO-1 gene decreases Ang II-mediated DNA damage [62].

HO-1 has been shown to play a vital role in protecting the kidney against various noxious stimuli. In the glycerol model of acute renal failure, increased release of heme proteins caused renal toxicity, whereas induction of HO-1 preserved renal function [63] and attenuated inflammatory molecules [14]. Conners et al [64] demonstrated that induction of HO-1 has an anti-inflammatory effect. This

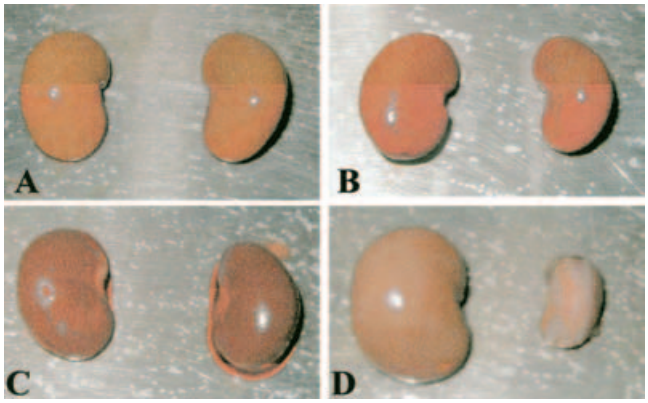

Fig. 8. Effect of treatment with CoPP (5 mg/100 g body wt/week) or SnMP (three doses of 5 mg/100 g body wt/week) on left (L, clipped) and right (R, non-clipped) kidney size. (*A*) Sham, (*B*) 2K1Cl control, (*C*) 2K1Cl treated with CoPP for three weeks, and (*D*) 2K1Cl pretreated with SnMP for three weeks.

finding has been substantiated by studies from other laboratories [65, 66]. Further, Wiesel et al have shown that the absence of HO-1 and presumably an increase in heme levels in the Goldblatt 1K1C model in mice leads to more severe renovascular hypertension and an increase in ischemic damage [67]. In ischemia/reperfusion injury, heat preconditioning or CoPP administration induced HO-1, preserved kidney graft function, and prevented postperfusion apoptosis after cold preservation [68]. The salutary effect of the enhancement of HO-1 activity has been attributed by some to the HO-1–mediated increase in GSH and extracellular superoxide dismutase levels, and the decrease in iNOS [59, 62, 69]. The decrease in oxidative stress has been shown to counteract vasoconstrictors, including ET-1 and PE [18, 24, 25, 62, 69].

CONCLUSION

We conclude that administration of CoPP to 2K1C rats significantly induced HO-1, which was associated with decreased cellular heme levels and COX-2 expression in the clipped kidney, increased PRA, and decreased plasma aldosterone levels. This study also shows that HO-1 upregulation in the 2K1C rats prevents the development of hypertension by decreasing cellular heme and increasing CO and bilirubin synthesis, which induces vasodilation (CO) and decreases oxidative stress (bilirubin). These pharmacologic strategies to regulate the heme-HO system may, in renovascular hypertension, have potential clinical significance.

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Reprint requests to Dr. Nader G. Abraham, Professor of Pharamacology, New York Medical College, Valhalla NY 10595.
E-mail: nader_abraham@nymc.edu

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